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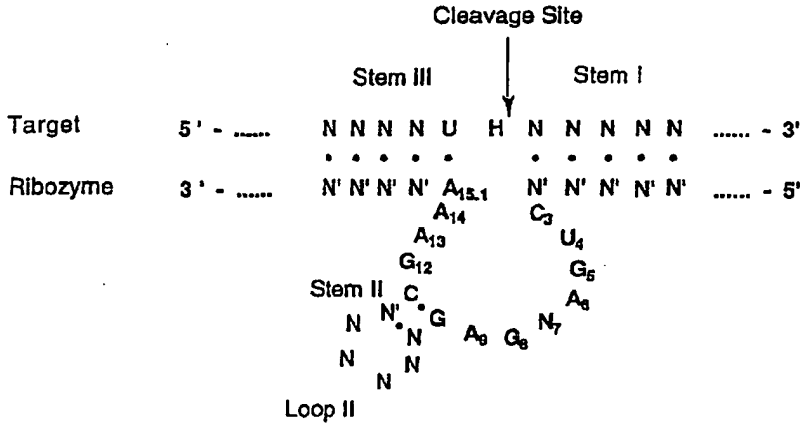
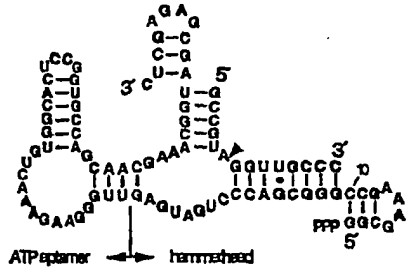
(57) Abstract

Nucleic acid molecules with new motifs having catalytic activity, methods of synthesis, and use thereof are also described.





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DESCRIPTION

Nucleic Acid Catalysts

Background of the Invention

This invention relates to nucleic acid molecules with catalytic activity and derivatives thereof.

The following is a brief description of enzymatic nucleic acid molecules. This summary is not meant to be complete but is provided only for understanding of the invention that follows. This summary is not an admission that all of the work described below is prior art to the claimed invention.

Enzymatic nucleic acid molecules (ribozymes) are nucleic acid molecules capable of catalyzing one or more of a variety of reactions, including the ability to repeatedly cleave other separate nucleic acid molecules in a nucleotide base sequence-specific manner. Such enzymatic nucleic acid molecules can be used, for example, to target virtually any RNA transcript (Zaug *et al.*, 324, *Nature* 429 1986; Cech, 260 *JAMA* 3030, 1988; and Jefferies *et al.*, 17 *Nucleic Acids Research* 1371, 1989).

Because of their sequence-specificity, *trans*-cleaving enzymatic nucleic acid molecules show promise as therapeutic agents for human disease (Usman & McSwiggen, 1995 *Ann. Rep. Med. Chem.* **30**, 285-294; Christoffersen and Marr, 1995 *J. Med. Chem.* **38**, 2023-2037). Enzymatic nucleic acid molecules can be designed to cleave specific RNA targets within the background of cellular RNA. Such a cleavage event renders the mRNA non-functional and abrogates protein expression from that RNA. In this manner, synthesis of a protein associated with a disease state can be selectively inhibited.

There are seven basic varieties of naturally-occurring enzymatic RNAs. Each can catalyze the hydrolysis of RNA phosphodiester bonds in *trans* (and thus can cleave other RNA molecules) under physiological conditions. In general, enzymatic nucleic acids act by first binding to a target RNA. Such binding occurs through the target binding portion of a enzymatic nucleic acid which is held in close proximity to an enzymatic portion of the molecule that acts to cleave the target RNA. Thus, the enzymatic nucleic acid first recognizes and then binds a target RNA through complementary base-pairing, and once bound to the correct site, acts enzymatically to cut the target RNA. Strategic cleavage of such a target RNA will destroy its ability to direct synthesis of an encoded protein. After an enzymatic nucleic acid has bound and cleaved its RNA target, it is released from that RNA to search for another target and can repeatedly bind and cleave new targets.

In addition, several *in vitro* selection (evolution) strategies (Orgel, 1979, *Proc. R. Soc. London,* B 205, 435) have been used to evolve new nucleic acid catalysts capable of catalyzing a variety of reactions, such as cleavage and ligation of phosphodiester linkages and amide linkages, (Joyce, 1989, *Gene,* 82, 83-87; Beaudry *et al.*,1992, *Science* 257, 635-641; Joyce, 1992, *Scientific American* 267, 90-97; Breaker *et al.*, 1994, *TIBTECH* 12, 268; Bartel *et al.*,1993, *Science* 261:1411-1418; Szostak, 1993, *TIBS* 17, 89-93; Kumar *et al.*, 1995, *FASEB J.*, 9, 1183; Breaker, 1996, *Curr. Op. Biotech.*, 7, 442).

The enzymatic nature of a ribozyme is advantageous over other technologies, since the effective concentration of ribozyme necessary to effect a therapeutic treatment is generally lower than that of an antisense oligonucleotide.

This advantage reflects the ability of the ribozyme to act enzymatically. Thus, a single ribozyme (enzymatic nucleic acid) molecule is able to cleave many molecules of target RNA. In addition, the ribozyme is a highly specific inhibitor, with the specificity of inhibition depending not only on the base-pairing mechanism of binding, but also on the mechanism by which the molecule inhibits the expression of the RNA to which it binds. That is, the inhibition is caused by cleavage of the RNA target and so specificity is defined as the ratio of the rate of cleavage of the targeted RNA over the rate of cleavage of non-targeted RNA. This cleavage mechanism is dependent upon factors additional to those involved in base-pairing. Thus, it is thought that the specificity of action of a ribozyme is greater than that of antisense oligonucleotide binding the same RNA site.

The development of ribozymes that are optimal for catalytic activity would contribute significantly to any strategy that employs RNA-cleaving ribozymes for the purpose of regulating gene expression. The hammerhead ribozyme functions with a catalytic rate (k_{cat}) of ~1 min^{-1} in the presence of saturating (10 mM) concentrations of Mg^{2+} cofactor. However, the rate for this ribozyme in Mg^{2+} concentrations that are closer to those found inside cells (0.5 - 2 mM) can be 10- to 100-fold slower. In contrast, the RNase P holoenzyme can catalyze pre-tRNA cleavage with a k_{cat} of ~30 min^{-1} under optimal assay conditions. An artificial 'RNA ligase' ribozyme has been shown to catalyze the corresponding self-modification reaction with a rate of ~100 min^{-1}. In addition, it is known that certain modified hammerhead ribozymes that have substrate binding arms made of DNA catalyze RNA cleavage with multiple turn-over rates that approach 100 min^{-1}. Finally, replacement

of a specific residue within the catalytic core of the hammerhead with certain nucleotide analogues gives modified ribozymes that show as much as a 10-fold improvement in catalytic rate. These findings demonstrate that ribozymes can promote chemical transformations with catalytic rates that are significantly greater than those displayed *in vitro* by most natural self-cleaving ribozymes. It is then possible that the structures of certain self-cleaving ribozymes may not be optimized to give maximal catalytic activity, or that entirely new RNA motifs could be made that display significantly faster rates for RNA phosphoester cleavage.

An extensive array of site-directed mutagenesis studies have been conducted with the hammerhead ribozyme to probe relationships between nucleotide sequence and catalytic activity. These systematic studies have made clear that most nucleotides in the conserved core of the hammerhead ribozyme cannot be mutated without significant loss of catalytic activity. In contrast, a combinatorial strategy that simultaneously screens a large pool of mutagenized ribozymes for RNAs that retain catalytic activity could be used more efficiently to define immutable sequences and to identify new ribozyme variants. Although similar *in vitro* selection experiments have been conducted with the hammerhead ribozyme (Nakamaye & Eckstein, 1994, *Biochemistry* 33, 1271; Long & Uhlenbeck, 1994, *Proc. Natl. Acad. Sci.*, 91, 6977; Ishizaka *et al.*, 1995, *BBRC* 214, 403), none of these efforts have successfully screened full-sized hammerhead ribozymes for all possible combinations of sequence variants that encompass the entire catalytic core.

The references cited above are distinct from the presently claimed invention since they do not disclose

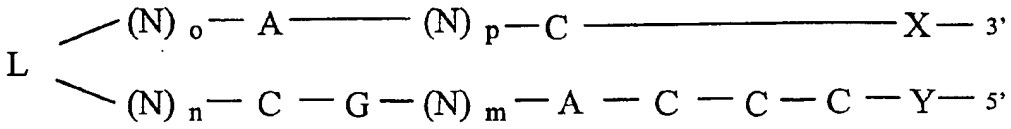
and/or contemplate the enzymatic nucleic acid molecules of the instant invention.

Summary of the Invention

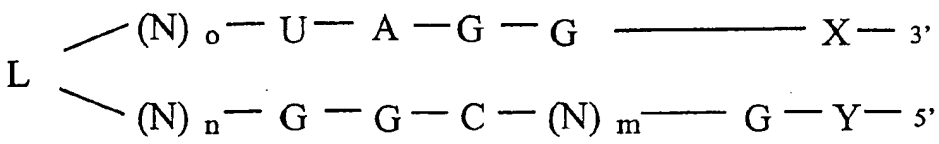
This invention relates to novel nucleic acid molecules with catalytic activity, which are particularly useful for cleavage of RNA or DNA. The nucleic acid catalysts of the instant invention are distinct from other nucleic acid catalysts known in the art. The nucleic acid catalysts of the instant invention do not share sequence homology with other known ribozymes. Specifically, nucleic acid catalysts of the instant invention are capable of catalyzing an intermolecular or intramolecular endonuclease reaction.

In a preferred embodiment, the invention features a nucleic acid molecule with catalytic activity having one of the formulae **I-V**:

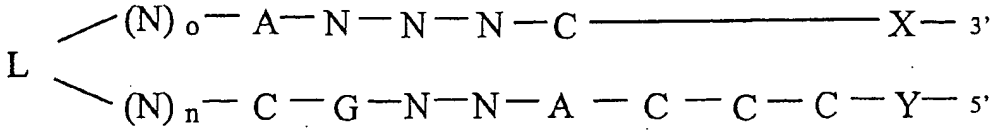
Formula I



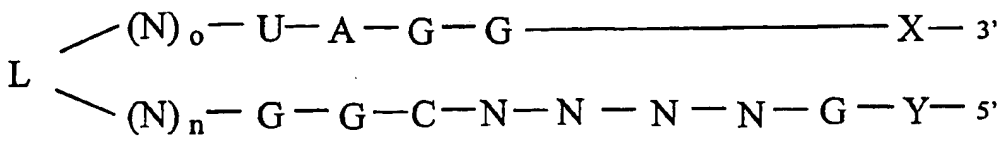
Formula II



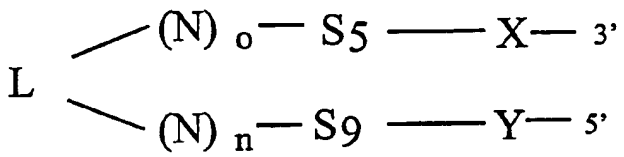
Formula III



Formula IV



Formula V



In each of the above formula, N represents independently a nucleotide or a non-nucleotide linker, which may be same or different; X and Y are independently oligonucleotides of length sufficient to stably interact (*e.g.*, by forming hydrogen bonds with complementary nucleotides in the target) with a target nucleic acid molecule (the target can be an RNA, DNA or RNA/DNA mixed polymers); o and n are integers greater than or equal to 1 and preferably less than about 100, wherein if $(N)_o$ and $(N)_n$ are nucleotides, (N)o and (N)n are optionally able to interact by hydrogen bond interaction; p and m are independently one of the integers 0, 1, 2, 3, 4 or 5; L is a linker which may be present or absent (*i.e.*, the molecule is assembled from two separate molecules), but when present, is a nucleotide and/or a non-nucleotide linker, which may be a single-stranded and/or double-stranded region; and _____ represents a chemical linkage(*e.g.* a phosphate ester linkage, amide linkage or others known in the art). A, C, U and G represent adenosine, cytidine, uridine and guanosine nucleotides, respectively. The nucleotides in the each of the formula I-V are unmodified or modified at the sugar, base, and/or phosphate as known in the art. S_5 is an oligonucleotide

containing a sequence selected from the group consisting of 5'-AUGUC-3', 5'-ACGUC-3', 5'-ACGGC-3', 5'-ACCUC-3', 5'-AAGGC-3', 5'-AUGGC-3', 5'-AUGCC-3', 5'-ACUCC-3', 5'-AUGAC-3', 5'-ACGAC-3', 5'-UUAGG-3', and 5'-CUAGG-3'; S_9 is an oligonucleotide containing a sequence selected from the group consisting of 5'-CCCAGUGCC-3', 5'-CCCAGUGCA-3', 5'-CCCAAUGCA-3', 5'-CCCAAUGCC-3', 5'-CCCAAUGCU-3', 5'-CCCAUAGCA-3', 5'-CCCAACGCA-3', 5'-CCCACCGCA-3', 5'-CCCAGAGCA-3', 5'-CCCAACGCU-3', 5'-CCCAUUGCA-3', 5'-GUAGACGGA-3', and 5'-GGUUUCGGG-3'. The nucleotides in the formula V are unmodified or modified at the sugar, base, and/or phosphate portions as known in the art.

In a preferred embodiment, the invention features nucleic acid molecules of any of Formulae I-IV further comprising a cytidine residue immediately 3' of $(N)_n$.

In yet another embodiment, the nucleotide linker (L) is a nucleic acid aptamer, such as an ATP aptamer, HIV Rev aptamer (RRE), HIV Tat aptamer (TAR) and others (for a review see Gold *et al.*, 1995, *Annu. Rev. Biochem.*, 64, 763; and Szostak & Ellington, 1993, in *The RNA World*, ed. Gesteland and Atkins, pp 511, CSH Laboratory Press). A "nucleic acid aptamer" as used herein is meant to indicate nucleic acid sequence capable of interacting with a ligand.. The ligand can be any natural or a synthetic molecule, including but not limited to a resin, metabolites, nucleosides, nucleotides, drugs, toxins, transition state analogs, peptides, lipids, proteins, aminoacids, nucleic acid molecules, hormones, carbohydrates, receptors, cells, viruses, bacteria and others.

In yet another embodiment, the non-nucleotide linker (L) is as defined herein.

The term "nucleotide" is used as recognized in the art to include natural bases, and modified bases well known in the art. Such bases are generally located at the 1' position of a sugar moiety. Nucleotide generally comprise a base, sugar and a phosphate group. The nucleotides can be unmodified or modified at the sugar, phosphate and/or base moeity, (see for example, Usman and McSwiggen, *supra;* Eckstein *et al.,* International PCT Publication No. WO 92/07065; Usman *et al.,* International PCT Publication No. WO 93/15187; all hereby incorporated by reference herein). There are several examples of modified nucleic acid bases known in the art and has recently been summarized by Limbach *et al.,* 1994, *Nucleic Acids Res.* 22, 2183. Some of the non-limiting examples of base modifications that can be introduced into enzymatic nucleic acids without significantly effecting their catalytic activity include, inosine, purine, pyridin-4-one, pyridin-2-one, phenyl, pseudouracil, 2, 4, 6-trimethoxy benzene, 3-methyluracil, dihydrouridine, naphthyl, aminophenyl, 5-alkylcytidines (*e.g.,* 5-methylcytidine), 5-alkyluridines (*e.g.,* ribothymidine), 5-halouridine (*e.g.,* 5-bromouridine) or 6-azapyrimidines or 6-alkylpyrimidines (*e.g.* 6-methyluridine) and others (Burgin *et al.,* 1996, *Biochemistry,* 35, 14090). By "modified bases" in this aspect is meant nucleotide bases other than adenine, guanine, cytosine and uracil at 1' position or their equivalents; such bases may be used within the catalytic core of the enzyme and/or in the substrate-binding regions.

In particular, the invention features modified ribozymes having a base substitution selected from pyridin-4-one, pyridin-2-one, phenyl, pseudouracil, 2, 4,

6-trimethoxy benzene, 3-methyluracil, dihydrouracil, naphthyl, 6-methyl-uracil and aminophenyl.

There are several examples in the art describing sugar and phosphate modifications that can be introduced into enzymatic nucleic acid molecules without significantly effecting catalysis and with significant enhancement in their nuclease stability and efficacy. Ribozymes are modified to enhance stability and/or enhance catalytic activity by modification with nuclease resistant groups, for example, 2'-amino, 2'-*C*-allyl, 2'-flouro, 2'-*O*-methyl, 2'-H, nucleotide base modifications (for a review see Usman and Cedergren, 1992 *TIBS* 17, 34; Usman *et al.*, 1994 *Nucleic Acids Symp. Ser.* 31, 163; Burgin *et al.*, 1996 *Biochemistry* 35, 14090). Sugar modification of enzymatic nucleic acid molecules have been extensively described in the art (see Eckstein *et al.*, *International Publication* PCT No. WO 92/07065; Perrault *et al. Nature* 1990, *344*, 565-568; Pieken *et al. Science* 1991, *253*, 314-317; Usman and Cedergren, *Trends in Biochem. Sci.* 1992, *17*, 334-339; Usman *et al. International Publication* PCT No. WO 93/15187; Sproat, *US Patent* No. 5,334,711 and Beigelman *et al.*, 1995 *J. Biol. Chem.* 270, 25702).

Such publications describe general methods and strategies to determine the location of incorporation of sugar, base and/or phosphate modifications and the like into ribozymes wihout inhibiting catalysis, and are incorporated by reference herein. In view of such teachings, similar modifications can be used as described herein to modifiy the nucleic acid catalysts of the instant invention.

In yet another embodiment, the non-nucleotide linker (L) is as defined herein. The term "non-nucleotide" as used herein include either abasic nucleotide, polyether,

polyamine, polyamide, peptide, carbohydrate, lipid, or polyhydrocarbon compounds. Specific examples include those described by Seela and Kaiser, *Nucleic Acids Res.* 1990, *18*:6353 and *Nucleic Acids Res.* 1987, *15*:3113; Cload and Schepartz, *J. Am. Chem. Soc.* 1991, *113*:6324; Richardson and Schepartz, *J. Am. Chem. Soc.* 1991, *113*:5109; Ma et al., *Nucleic Acids Res.* 1993, *21*:2585 and *Biochemistry* 1993, *32*:1751; Durand et al., *Nucleic Acids Res.* 1990, *18*:6353; McCurdy et al., *Nucleosides & Nucleotides* 1991, *10*:287; Jschke et al., *Tetrahedron Lett.* 1993, *34*:301; Ono et al., *Biochemistry* 1991, *30*:9914; Arnold *et al.*, International Publication No. WO 89/02439; Usman *et al.*, International Publication No. WO 95/06731; Dudycz *et al.*, International Publication No. WO 95/11910 and Ferentz and Verdine, *J. Am. Chem. Soc.* 1991, *113*:4000, all hereby incorporated by reference herein. Thus, in a preferred embodiment, the invention features an enzymatic nucleic acid molecule having one or more non-nucleotide moieties, and having enzymatic activity to cleave an RNA or DNA molecule. By the term "non-nucleotide" is meant any group or compound which can be incorporated into a nucleic acid chain in the place of one or more nucleotide units, including either sugar and/or phosphate substitutions, and allows the remaining bases to exhibit their enzymatic activity. The group or compound is abasic in that it does not contain a commonly recognized nucleotide base, such as adenosine, guanine, cytosine, uracil or thymine. The terms "abasic" or "abasic nucleotide" as used herein encompass sugar moieties lacking a base or having other chemical groups in place of base at the 1' position.

In preferred embodiments, the enzymatic nucleic acid includes one or more stretches of RNA, which provide the

enzymatic activity of the molecule, linked to the non-nucleotide moiety. The necessary RNA components are known in the art, see, *e.g.*, Usman, *supra*. By RNA is meant a molecule comprising at least one ribonucleotide residue.

As the term is used in this application, non-nucleotide-containing enzymatic nucleic acid means a nucleic acid molecule that contains at least one non-nucleotide component which replaces a portion of a ribozyme, *e.g.*, but not limited to, a double-stranded stem, a single-stranded "catalytic core" sequence, a single-stranded loop or a single-stranded recognition sequence. These molecules are able to cleave (preferably, repeatedly cleave) separate RNA or DNA molecules in a nucleotide base sequence specific manner. Such molecules can also act to cleave intramolecularly if that is desired. Such enzymatic molecules can be targeted to virtually any RNA transcript.

By the phrase "nucleic acid catalyst" is meant a nucleic acid molecule capable of catalyzing (altering the velocity and/or rate of) a variety of reactions including the ability to repeatedly cleave other separate nucleic acid molecules (endonuclease activity) in a nucleotide base sequence-specific manner. Such a molecule with endonuclease activity may have complementarity in a substrate binding region (*e.g.* X and Y in formulae I-V) to a specified gene target, and also has an enzymatic activity that specifically cleaves RNA or DNA in that target. That is, the nucleic acid molecule with endonuclease activity is able to intramolecularly or intermolecularly cleave RNA or DNA and thereby inactivate a target RNA or DNA molecule. This complementarity functions to allow sufficient hybridization of the enzymatic RNA molecule to the target RNA or DNA to allow the cleavage to occur. 100% complementarity is preferred,

but complementarity as low as 50-75% may also be useful in this invention. The nucleic acids may be modified at the base, sugar, and/or phosphate groups. The term enzymatic nucleic acid is used interchangeably with phrases such as ribozymes, catalytic RNA, enzymatic RNA, catalytic DNA, nucleozyme, DNAzyme, RNA enzyme, endoribonuclease, endonuclease, minizyme, leadzyme, oligozyme or DNA enzyme. All of these terminologies describe nucleic acid molecules with enzymatic activity.

By "nucleic acid molecule" as used herein is meant a molecule comprising nucleotides. The nucleic acid can be composed of modified or unmodified nucleotides or non-nucleotides or various mixtures and combinations thereof.

By "complementarity" is meant a nucleic acid that can form hydrogen bond(s) with other RNA sequence by either traditional Watson-Crick or other non-traditional types (for example, Hoogsteen type) of base-paired interactions.

By "oligonucleotide" as used herein, is meant a molecule comprising two or more nucleotides.

The specific enzymatic nucleic acid molecules described in the instant application are not limiting in the invention and those skilled in the art will recognize that all that is important in an enzymatic nucleic acid molecule of this invention is that it has a specific substrate binding site (*e.g.*, X and/or Y of Formulae 1-V above) which is complementary to one or more of the target nucleic acid regions, and that it have nucleotide sequences within or surrounding that substrate binding site which impart a nucleic acid cleaving activity to the molecule.

The invention provides a method for producing a class of enzymatic cleaving agents which exhibit a high degree of specificity for the nucleic acid sequence of a desired target. The enzymatic nucleic acid molecule is preferably

targeted to a highly conserved sequence region of a target such that specific diagnosis and/or treatment of a disease or condition can be provided with a single enzymatic nucleic acid. Such enzymatic nucleic acid molecules can be delivered exogenously to specific cells as required. In the preferred hammerhead motif the small size (less than 60 nucleotides, preferably between 30-40 nucleotides in length) of the molecule allows the cost of treatment to be reduced.

Therapeutic ribozymes must remain stable within cells until translation of the target RNA has been inhibited long enough to reduce the levels of the undesirable protein. This period of time varies between hours to days depending upon the disease state. Clearly, ribozymes must be resistant to nucleases in order to function as effective intracellular therapeutic agents. Improvements in the chemical synthesis of RNA (Wincott *et al.*, 1995 *Nucleic Acids Res.* 23, 2677; incorporated by reference herein) have expanded the ability to modify ribozymes to enhance their nuclease stability.

By "enzymatic portion" is meant that part of the ribozyme essential for cleavage of an RNA substrate.

By "substrate binding arm" is meant that portion of a ribozyme which is complementary to (*i.e.*, able to base-pair with) a portion of its substrate. Generally, such complementarity is 100%, but can be less if desired. For example, as few as 10 bases out of 14 may be base-paired. Such arms are shown generally in Figure 1A and as X and/or Y in Formulae I-V. That is, these arms contain sequences within a ribozyme which are intended to bring ribozyme and target RNA together through complementary base-pairing interactions.

In a preferred embodiment, the invention provides a method for producing a class of enzymatic cleaving agents which exhibit a high degree of specificity for the nucleic acid of a desired target. Such enzymatic nucleic acid molecules can be delivered exogenously to specific cells as required. Alternatively, the ribozymes can be expressed from DNA/RNA vectors that are delivered to specific cells.

The enzymatic nucleic acid molecules of the instant invention can also be expressed within cells from eukaryotic promoters (*e.g.*, Izant and Weintraub, 1985 *Science* 229, 345; McGarry and Lindquist, 1986 *Proc. Natl. Acad. Sci.* USA 83, 399; SullengerScanlon *et al.*, 1991, *Proc. Natl. Acad. Sci. USA*, 88, 10591-5; Kashani-Sabet *et al.*, 1992 *Antisense Res. Dev.*, 2, 3-15; Dropulic *et al.*, 1992 *J. Virol*, 66, 1432-41; Weerasinghe *et al.*, 1991 *J. Virol*, **65**, 5531-4; Ojwang *et al.*, 1992 *Proc. Natl. Acad. Sci. USA* 89, 10802-6; Chen *et al.*, 1992 *Nucleic Acids Res.*, 20, 4581-9; Sarver *et al.*, 1990 *Science* 247, 1222-1225; Thompson *et al.*, 1995 *Nucleic Acids Res.* 23, 2259). Those skilled in the art realize that any nucleic acid can be expressed in eukaryotic cells from the appropriate DNA/RNA vector. The activity of such nucleic acids can be augmented by their release from the primary transcript by a ribozyme (Draper *et al.*, PCT WO93/23569, and Sullivan *et al.*, PCT WO94/02595; Ohkawa *et al.*, 1992 *Nucleic Acids Symp. Ser.*, 27, 15-6; Taira *et al.*, 1991, *Nucleic Acids Res.*, 19, 5125-30; Ventura *et al.*, 1993 *Nucleic Acids Res.*, 21, 3249-55; Chowrira *et al.*, 1994 *J. Biol. Chem.* 269, 25856; hereby incorporated in their totality by reference herein).

By "consists essentially of" is meant that the active ribozyme contains an enzymatic center or core equivalent

to those in the examples, and binding arms able to bind target nucleic acid molecules such that cleavage at the target site occurs. Other sequences may be present which do not interfere with such cleavage.

Thus, in one aspect, the invention features ribozymes that inhibit gene expression and/or cell proliferation. These chemically or enzymatically synthesized nucleic acid molecules contain substrate binding domains that bind to accessible regions of specific target nucleic acid molecules. The nucleic acid molecules also contain domains that catalyze the cleavage of target. Upon binding, the enzymatic nucleic acid molecules cleave the target molecules, preventing for example, translation and protein accumulation. In the absence of the expression of the target gene, cell proliferation, for example, is inhibited.

In a preferred embodiment, the enzymatic nucleic acid molecules are added directly, or can be complexed with cationic lipids, packaged within liposomes, or otherwise delivered to smooth muscle cells. The RNA or RNA complexes can be locally administered to relevant tissues through the use of a catheter, infusion pump or stent, with or without their incorporation in biopolymers. Using the methods described herein, other enzymatic nucleic acid molecules that cleave target nucleic acid may be derived and used as described above. Specific examples of nucleic acid catalysts of the instant invention are provided below in the Tables and figures.

Sullivan, *et al., supra*, describes the general methods for delivery of enzymatic RNA molecules. Ribozymes may be administered to cells by a variety of methods known to those familiar to the art, including, but not restricted to, encapsulation in liposomes, by iontophoresis, or by incorporation into other vehicles, such as

hydrogels, cyclodextrins, biodegradable nanocapsules, and bioadhesive microspheres. For some indications, ribozymes may be directly delivered *ex vivo* to cells or tissues with or without the aforementioned vehicles. Alternatively, the RNA/vehicle combination is locally delivered by direct injection or by use of a catheter, infusion pump or stent. Other routes of delivery include, but are not limited to, intravascular, intramuscular, subcutaneous or joint injection, aerosol inhalation, oral (tablet or pill form), topical, systemic, ocular, intraperitoneal and/or intrathecal delivery. More detailed descriptions of ribozyme delivery and administration are provided in Sullivan *et al.*, supra and Draper *et al.*, *supra* which have been incorporated by reference herein.

In another aspect of the invention, enzymatic nucleic acid molecules that cleave target molecules are expressed from transcription units inserted into DNA or RNA vectors. The recombinant vectors are preferably DNA plasmids or viral vectors. Ribozyme expressing viral vectors could be constructed based on, but not limited to, adeno-associated virus, retrovirus, adenovirus, or alphavirus. Preferably, the recombinant vectors capable of expressing the ribozymes are delivered as described above, and persist in target cells. Alternatively, viral vectors may be used that provide for transient expression of ribozymes. Such vectors might be repeatedly administered as necessary. Once expressed, the ribozymes cleave the target mRNA. Delivery of ribozyme expressing vectors could be systemic, such as by intravenous or intramuscular administration, by administration to target cells ex-planted from the patient followed by reintroduction into the patient, or by any other means that would allow for introduction into the desired target cell (for a review see Couture and Stinchcomb, 1996, *TIG.*, 12, 510).

In a preferred embodiment, an expression vector comprising nucleic acid sequence encoding at least one of the nucleic acid catalyst of the instant invention is disclosed. The nucleic acid sequence encoding the nucleic acid catalyst of the instant invention is operable linked in a manner which allows expression of that nucleic acid molecule.

In one embodiment, the expression vector comprises: a transcription initiation region (*e.g.*, eukaryotic pol I, II or III initiation region); b) a transcription termination region (*e.g.*, eukaryotic pol I, II or III termination region); c) a gene encoding at least one of the nucleic acid catalyst of the instant invention; and wherein said gene is operably linked to said initiation region and said termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule. The vector may optionally include an open reading frame (ORF) for a protein operably linked on the 5' side or the 3'-side of the gene encoding the nucleic acid catalyst of the invention; and/or an intron (intervening sequences).

By "patient" is meant an organism which is a donor or recipient of explanted cells or the cells themselves. "Patient" also refers to an organism to which enzymatic nucleic acid molecules can be administered. Preferably, a patient is a mammal or mammalian cells. More preferably, a patient is a human or human cells.

By "vectors" is meant any nucleic acid- and/or viral-based technique used to deliver a desired nucleic acid.

Another means of accumulating high concentrations of a ribozyme(s) within cells is to incorporate the ribozyme-encoding sequences into a DNA or RNA expression vector. Transcription of the ribozyme sequences are driven from a promoter for eukaryotic RNA polymerase I (pol I), RNA

polymerase II (pol II), or RNA polymerase III (pol III). Transcripts from pol II or pol III promoters will be expressed at high levels in all cells; the levels of a given pol II promoter in a given cell type will depend on the nature of the gene regulatory sequences (enhancers, silencers, etc.) present nearby. Prokaryotic RNA polymerase promoters are also used, providing that the prokaryotic RNA polymerase enzyme is expressed in the appropriate cells (Elroy-Stein and Moss, 1990 *Proc. Natl. Acad. Sci. U S A*, 87, 6743-7; Gao and Huang 1993 *Nucleic Acids Res.*, 21, 2867-72; Lieber et al., 1993 *Methods Enzymol.*, 217, 47-66; Zhou et al., 1990 *Mol. Cell. Biol.*, 10, 4529-37). Several investigators have demonstrated that ribozymes expressed from such promoters can function in mammalian cells (e.g. Kashani-Sabet et al., 1992 *Antisense Res. Dev.*, 2, 3-15; Ojwang et al., 1992 *Proc. Natl. Acad. Sci. U S A*, 89, 10802-6; Chen *et al.*, 1992 *Nucleic Acids Res.*, 20, 4581-9; Yu et al., 1993 *Proc. Natl. Acad. Sci. U S A*, 90, 6340-4; L'Huillier *et al.*, 1992 *EMBO J.* 11, 4411-8; Lisziewicz *et al.*, 1993 *Proc. Natl. Acad. Sci. U. S. A.*, 90, 8000-4;Thompson *et al.*, 1995 *Nucleic Acids Res.* 23, 2259; Sullenger & Cech, 1993, *Science*, 262, 1566). The above ribozyme transcription units can be incorporated into a variety of vectors for introduction into mammalian cells, including but not restricted to, plasmid DNA vectors, viral DNA vectors (such as adenovirus or adeno-associated virus vectors), or viral RNA vectors (such as retroviral or alphavirus vectors) (for a review see Couture and Stinchcomb, *1996, supra)*.

In a preferred embodiment, the invention features a method of synthesis of enzymatic nucleic acid molecules of instant invention which follows the procedure for normal

chemical synthesis of RNA as described in Usman *et al.*, 1987 *J. Am. Chem. Soc.*, 109, 7845; Scaringe *et al.*, 1990 *Nucleic Acids Res.*, 18, 5433; and Wincott *et al.*, 1995 *Nucleic Acids Res.* 23, 2677-2684 and makes use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end, and phosphoramidites at the 3'-end. Small scale synthesis were conducted on a 394 Applied Biosystems, Inc. synthesizer using a modified 2.5 µmol scale protocol with a 5 min coupling step for alkylsilyl protected nucleotides and 2.5 min coupling step for 2'-*O*-methylated nucleotides. Table **II** outlines the amounts, and the contact times, of the reagents used in the synthesis cycle. A 6.5-fold excess (163 µL of 0.1 M = 16.3 µmol) of phosphoramidite and a 24-fold excess of *S*-ethyl tetrazole (238 µL of 0.25 M = 59.5 µmol) relative to polymer-bound 5'-hydroxyl is used in each coupling cycle. Average coupling yields on the 394 Applied Biosystems, Inc. synthesizer, determined by colorimetric quantitation of the trityl fractions, is 97.5-99%. Other oligonucleotide synthesis reagents for the 394 Applied Biosystems, Inc. synthesizer : detritylation solution was 2% TCA in methylene chloride (ABI); capping was performed with 16% *N*-methyl imidazole in THF (ABI) and 10% acetic anhydride/10% 2,6-lutidine in THF (ABI); oxidation solution was 16.9 mM I_2, 49 mM pyridine, 9% water in THF (Millipore). B & J Synthesis Grade acetonitrile is used directly from the reagent bottle. *S*-Ethyl tetrazole solution (0.25 M in acetonitrile) is made up from the solid obtained from American International Chemical, Inc.

In a preferred embodiment, deprotection of the chemically synthesized nucleic acid catalysts of the invention is performed as follows. The polymer-bound oligoribonucleotide, trityl-off, is transferred from the

synthesis column to a 4mL glass screw top vial and suspended in a solution of methylamine (MA) at 65 °C for 10 min. After cooling to -20 °C, the supernatant is removed from the polymer support. The support is washed three times with 1.0 mL of EtOH:MeCN:H_2O/3:1:1, vortexed and the supernatant is then added to the first supernatant. The combined supernatants, containing the oligoribonucleotide, are dried to a white powder.

The base-deprotected oligoribonucleotide is resuspended in anhydrous TEA•HF/NMP solution (250 µL of a solution of 1.5mL *N*-methylpyrrolidinone, 750 µL TEA and 1.0 mL TEA•3HF to provide a 1.4M HF concentration) and heated to 65°C for 1.5 h. The resulting, fully deprotected, oligomer is quenched with 50 mM TEAB (9 mL) prior to anion exchange desalting.

For anion exchange desalting of the deprotected oligomer, the TEAB solution is loaded on to a Qiagen 500® anion exchange cartridge (Qiagen Inc.) that is prewashed with 50 mM TEAB (10 mL). After washing the loaded cartridge with 50 mM TEAB (10 mL), the RNA is eluted with 2 M TEAB (10 mL) and dried down to a white powder. The average stepwise coupling yields are generally >98% (Wincott *et al.*, 1995 *Nucleic Acids Res.* 23, 2677-2684).

Ribozymes of the instant invention are also synthesized from DNA templates using bacteriophage T7 RNA polymerase (Milligan and Uhlenbeck, 1989, *Methods Enzymol.* 180, 51).

Ribozymes are purified by gel electrophoresis using general methods or are purified by high pressure liquid chromatography (HPLC; See Wincott *et al.*, *supra*) the totality of which is hereby incorporated herein by reference) and are resuspended in water.

In another preferred embodiment, catalytic activity of the molecules described in the instant invention can be optimized as described by Draper et al., *supra*. The details will not be repeated here, but include altering the length of the ribozyme binding arms, or chemically synthesizing ribozymes with modifications (base, sugar and/or phosphate) that prevent their degradation by serum ribonucleases and/or enhance their enzymatic activity (see *e.g.*, Eckstein *et al.*, International Publication No. WO 92/07065; Perrault *et al.*, 1990 *Nature* 344, 565; Pieken et al., 1991 *Science* 253, 314; Usman and Cedergren, 1992 *Trends in Biochem. Sci.* 17, 334; Usman *et al.*, International Publication No. WO 93/15187; and Rossi *et al.*, International Publication No. WO 91/03162; Sproat, US Patent No. 5,334,711; and Burgin *et al.*, *supra;* all of these describe various chemical modifications that can be made to the base, phosphate and/or sugar moieties of enzymatic RNA molecules). Modifications which enhance their efficacy in cells, and removal of bases from stem loop structures to shorten RNA synthesis times and reduce chemical requirements are desired. (All these publications are hereby incorporated by reference herein.).

By "enhanced enzymatic activity" is meant to include activity measured in cells and/or *in vivo* where the activity is a reflection of both catalytic activity and ribozyme stability. In this invention, the product of these properties is increased or not significantly (less that 10 fold) decreased *in vivo* compared to an all RNA ribozyme.

In yet another preferred embodiment, nucleic acid catalysts having chemical modifications which maintain or enhance enzymatic activity is provided. Such nucleic acid

is also generally more resistant to nucleases than unmodified nucleic acid. Thus, in a cell and/or *in vivo* the activity may not be significantly lowered. As exemplified herein such ribozymes are useful in a cell and/or *in vivo* even if activity over all is reduced 10 fold (Burgin *et al.*, 1996, *Biochemistry*, 35, 14090). Such ribozymes herein are said to "maintain" the enzymatic activity on all RNA ribozyme.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Description of the Preferred Embodiments

The drawings will first briefly be described.

Drawings:

Figure 1 A) is a diagrammatic representation of the hammerhead ribozyme domain known in the art. Stem II can be ≥ 2 base-pair long. Each N is independently any base or non-nucleotide as used herein. B) is a schematic representation of an ATP-dependent ribozyme (H3).

Figure 2 A-C are diagrammatic representations of self-cleaving ribozyme constructs. The arrow head indicates the site of cleavage. N_5 and N9 represent the regions of randomization. N_9, nine nucleotides in the region from position16-24 were randomized. N_5, five nucleotides in the region from position 59-63 were randomized.

Figure 3 is a schemmatic representation of a non-limiting *in vitro* selection strategy (allosteric delay) used to evolve nucleic acid catalysts. RT-PCR indicates reverse transcription (RT) and polymerase chain reaction (PCR). This step involves the conversion of RNA into complementary DNA using reverse transcriptase enzyme

followed by PCR amplification to generate a double stranded DNA template for further rounds of selection.

Figure 4 shows a comparison of the yields of RNA self-cleavage from AD-H2 and the generation 4 (G4) and generation 6 (G6) RNA pools in buffer B. Shaded and open bars depict the fraction of precursor cleaved in the presence and absence of ATP, respectively.

Figure 5 Sequences of individual RNAs that represent two new classes of self-cleaving ribozymes. **A**: Class I ribozymes, that were cloned from populations G6 (generation 6), G7-ATP (generation 7, without ATP), or G9-low (generation 9 in low magnesium concentration) as indicated, are defined by the presence of similar sequences in the regions that correspond to the N_9 and N_5 random-sequence domains of AD-H2. Each member of class I has also acquired a single G to C mutation at nucleotide 28 of the mutagenized AD-H2 construct. **B**: Class II ribozymes were represented only once (v1) in 34 sequences analyzed from G6. This individual has acquired two additional mutations at nucleotides 35 (A to G) and 37 (U to A) in the aptamer domain of mutagenized AD-H2. An additional class II ribozyme (v2) was isolated from the G9-low population. This variant has the same two mutations as class II v1, but has also acquired a C to A mutation at position 51, and a G deletion at position 32, each relative to the mutagenized AD-H2 construct.

Figure 6 Sequence conservation in the N_9 and N_5 domains among 24 individual class I ribozymes. The frequency of sequence variation compared to clone v2 is plotted as stacked bars, where component bars indicate the contribution to sequence variation for individual nucleotides.

Figure 7 Sequence and possible secondary structure of the constructs 'v2 trans' used to assess the catalytic activity of separate enzyme and substrate domains of a class I ribozyme. Nucleotides in the substrate-binding arms have been altered to complement the corresponding substrate RNA. Encircled nucleotides match those nucleotides that are characteristic of the class I ribozyme 'v2' (Figure 5A). Arrowhead identifies the new site of cleavage.

Examples

The following are non-limiting examples showing the selection, isolation, synthesis and activity of enzymatic nucleic acids of the instant invention.

The development of nucleic acid catalysts that are optimal for catalytic activity would contribute significantly to any strategy that employs nucleic acid cleaving ribozymes for the purpose of regulating gene expression. The hammerhead ribozyme functions with a catalytic rate (k_{cat}) of ~1 min^{-1} in the presence of saturating (10 mM) concentrations of Mg^{2+} cofactor. However, the rate for this ribozyme in Mg^{2+} concentrations that are closer to those found inside cells (0.5 - 2 mM) may be 10- to 100-fold slower. In contrast, the RNase P holoenzyme is beleived to catalyze pre-tRNA cleavage with a k_{cat} of ~30 min^{-1} under optimal assay conditions. An artificial 'RNA ligase' ribozyme has been shown to catalyze the corresponding self-modification reaction with a rate of ~100 min^{-1} (Ekland et al., 1995, *Science*, 269, 364). Finally, replacement of a specific residue within the catalytic core of the hammerhead with certain nucleotide analogues gives modified ribozymes that show as much as a 10-fold improvement in catalytic rate (Burgin et al., 1996, *supra*). These findings demonstrate that

ribozymes can promote chemical transformations with catalytic rates that are significantly greater than those displayed *in vitro* by most natural self-cleaving ribozymes. It is then possible that the structures of certain self-cleaving ribozymes may not be optimized to give maximal catalytic activity, or that entirely new RNA motifs could be made that display significantly faster rates for RNA phosphoester cleavage.

An extensive array of site-directed mutagenesis studies have been conducted with the hammerhead to probe relationships between nucleotide sequence and catalytic activity. These systematic studies have made clear that most nucleotides in the conserved core of the hammerhead ribozyme (Forster & Symons, 1987, *Cell*, 49, 211) cannot be mutated without significant loss of catalytic activity. In contrast, a combinatorial strategy that simultaneously screens a large pool of mutagenized ribozymes for RNAs that retain catalytic activity could be used more efficiently to define immutable sequences and to identify new ribozyme variants (Breaker, 1997, *supra*). For example, Joseph and Burke (1993; *J. Biol. Chem.*, 268, 24515) have used an *in vitro* selection approach to rapidly screen for sequence variants of the 'hairpin' self-cleaving RNA that show improved catalytic activity. This approach was successful in identifying two mutations in the hairpin ribozyme that together give a 10-fold improvement in catalytic rate. Although similar *in vitro* selection experiments have been conducted with the hammerhead ribozyme (Nakamaye & Eckstein, 1994, *supra*; Long & Uhlenbeck, 1994, supra; Ishizaka et al., 1995, *supra*), none of these efforts have successfully screened full-sized hammerhead ribozymes for all possible combinations

of sequence variants that encompass the entire catalytic core.

Applicant employed *in vitro* selection strategy to comprehensively test whether the natural consensus sequence for the core of the hammerhead ribozyme produces maximal catalytic rates, or whether sequence variants of this ribozyme could catalyze endonuclease reaction similar to or better than the hammerhead ribozyme.

A selection method for self-cleaving ribozymes makes use of the gel-mobility shift that occurs when full-length ribozyme precursors are fragmented and separated by polyacrylamide gel electrophoresis (PAGE). The hammerhead ribozyme can efficiently promote its own cleavage in the presence of the Mg^{2+}, a metal ion that serves as a cofactor for natural self-cleaving ribozymes and that is also a required component of *in vitro* transcription reactions. As a result, a significant portion of the ribozyme transcripts will self-cleave during preparation by *in vitro* transcription. The ribozyme precursor cannot be isolated from the spurious and unwanted RNA products that are typical of *in vitro* transcription, without losing the portion of precursors that have cleaved during the transcription reaction. Moreover, the best ribozymes present in a mutagenized or random-sequence pool can cleave during transcription, thereby creating a significant impediment to the *in vitro* selection of self-cleaving RNAs. Although ribozymes that cleave during transcription can be recovered by PAGE as part of the *in vitro* selection process, this approach can easily lead to the recovery of smaller 'selfish' RNAs that are not catalytic, but that have an electrophoretic mobility that serendipitously correspond to the cleaved ribozymes (Nakamaye & Eckstein, 1994). In addition, if the selection

process relies on the isolation of ribozymes that cleave during preparation, there is no possibility for selection under alternative reaction conditions that are not compatible with *in vitro* transcription.

Example 1: Preparation of intact self-cleaving ribozymes using 'allosteric delay'

Applicant has overcome the problem of ribozyme self-destruction during preparative transcription by making use of 'allosteric ribozyme' constructs that are not cleaved during transcription, but that remain highly active upon purification. Applicant used an ATP-dependent allosteric ribozyme H3 (Figure 1B; Tang and Breaker, 1997, submitted for publication) in which a hammerhead ribozyme was joined to an RNA aptamer (Sassanfar & Szostak, 1993, *Nature* 364, 550) that binds adenosine or any of its 5'-phosphorylated derivatives. H3 cleaves separate substrate molecules with a catalytic rate that is highly-dependent on the presence of adenosine of ATP. This conjoined aptamer/ribozyme construct experiences a ~170-fold reduction in catalytic activity upon addition of 1 mM nucleoside or nucleotide effector.

One of the ribozyme constructs designed by the applicant (Figure 2A; AD-H1) was designed to be analogous to H3, except that the ribozyme and substrate domains in the new construct are contained within a single molecule. Preparation of AD-H1 in a 3-hr *in vitro* transcription reaction resulted in almost complete inhibition of ribozyme function and, as a result, near complete preservation of the unimolecular ribozyme precursor. All transcription reactions conducted in this study initially contained 2 mM of each of the four ribonucleoside 5'-triphosphates (NTPs). Therefore, the ATP concentration during transcription far exceeds the K_D of the ATP-specific

aptamer (10 μM), and also exceeds the ATP concentration that was needed to give maximal inhibition of the allosteric ribozyme H3. The purified AD-H1 ribozyme, however, is highly active when allowed to react in the absence of ATP. AD-H1 is equally active when incubated in 50 mM Tris-HCl (pH 7.5 at 23°C) and 20 mM $MgCl_2$ (buffer B) or in the transcription buffer (buffer A) in the absence of NTPs. The catalytic rate for AD-H1 (Table 1) is ~3-fold slower than the rate for a similar hammerhead ribozyme without the appended aptamer domain. However, the catalytic activity of AD-H1 is significantly reduced in the presence of 1 mM ATP or when incubated with the same concentration of NTPs used for *in vitro* transcription. As a result, the timing of allosteric ribozyme function can be delayed in the presence of specific allosteric effector molecules, thereby allowing the preparation of intact self-cleaving ribozymes by *in vitro* transcription.

In vitro selection of self-cleaving RNAs from the rAD-H2 pool

Applicant has made use of this 'allosteric delay' strategy in its efforts to probe the catalytic fitness of the hammerhead ribozyme using *in vitro* selection. The selection was begun with an initial RNA pool (Generation 0; G0), termed 'rAD-H2' (Figure 2C), in which 14 nucleotides of the catalytic core were made random. The randomized region of rAD-H2 is divided into two domains that Applicant identified as 'N_9' and 'N_5'. The G0 RNA pool contained 3 x 10^{13} molecules, corresponding to an average representation of ~100,000 copies for each possible RNA sequence variant. The rAD-H2 pool was subjected to six successive rounds of *in vitro* selection (see the scheme in Figure 3), which proceeded by first isolating *in vitro* transcribed RNA precursors by PAGE, then by incubating the

RNA pool in the absence of ATP and recovering cleaved RNAs by a gel-mobility shift protocol. Robust self-cleaving activity of the RNA pool was detected after four rounds of selection (Generation 4;G4), with some improvement in the catalytic activity observed with the RNA pool from G6 (Figure 4).

By "randomized region" is meant a region of completely random sequence and/or partially random sequence. By completely random sequence is meant a sequence wherein theoretically there is equal representation of A, T, G and C nucleotides or modified derivatives thereof, at each position in the sequence. By partially random sequence is meant a sequence wherein there is an unequal representation of A, T, G and C nucleotides or modified derivatives thereof, at each position in the sequence. A partially random sequence can therefore have one or more positions of complete randomness and one or more positions with defined nucleotides

Applicant found that 15 of the 38 clones examined from the G6 pool matched the sequence of AD-H2, while 15 additional clones carried a single U to C change at position 21 (Figure 2B; corresponding to position 7 of the hammerhead core in Figure 1A). The identity of this nucleotide is variable in natural hammerhead isolates, and ribozymes with mutations at this position are known to be active *in vitro*. The k_{obs} values for AD-H2 with U or C were 0.068 min^{-1} and 0.041 min^{-1}, respectively, which are consistent with the k_{obs} of 0.047 min^{-1} determined for the ensemble of ribozymes that comprise the G6 pool (Table 1).

Further characterization of the dominant ribozymes reveals that the C and U variants of AD-H2 experience a ~100-fold reduction in catalytic rate in the presence of

1 mM ATP (Table 1). Although AD-H2 experiences dramatic allosteric inhibition, the G4 and G6 RNA populations display considerable activity despite the presence of 1 mM ATP (Figure 4). This finding suggests that a significant portion of the selected RNAs do not conform to the allosteric ribozyme design and are not inhibited by ATP. Indeed, N_9 and N_5 domains of the remaining eight clones from G6 do not match the hammerhead consensus sequence, but can be grouped into two new classes of Mg^{2+}-dependent self-cleaving ribozymes (Figure 5). Individuals from class I and an individual from class II display k_{obs} values that match those of the winning hammerhead ribozymes in the absence of ATP, but show no inhibition (class I) or only slight inhibition (class II) in the presence of 1 mM ATP (Table 1).

Although ribozymes that carry the natural hammerhead consensus sequence dominate the selection that used 20 mM Mg^{2+} as cofactor, Applicant wanted to assess the catalytic fitness of the ribozymes under conditions that simulate the ionic strength and Mg^{2+} concentrations of cells (buffer C; 50 mM Tris-HCl (pH 7.5 at 23□C), 250 mM KCl and 2 mM Mg^{2+}). To this end, G5 RNA pool was used to carry out additional rounds of selection using buffer C for the ribozyme selection reaction. The population that was isolated after four rounds using reduced Mg^{2+} concentrations (G9-low) displays a rate for self-cleavage of 0.012 min^{-1} in buffer C. Sequence analysis of 8 full-length clones from the G9-low pool revealed 6 sequences that conform to class I ribozyme variants (Figure 5A), while the hammerhead and class II (Figure 5B) variant ribozymes were represented by only one individual each. Dominance by class I ribozymes in the low-concentration Mg^{2+} selection is consistent with the fact that these

ribozymes show 2- to 5-fold higher rates in buffer C compared to the AD-H2 ribozyme variants.

Characterization of class I variant ribozymes

The two variants of the AD-H2 ribozyme dominate the G6 RNA pool, accounting for nearly 80% of the RNA population, while class I variants represent less that 20% of the population. In order to more efficiently accumulate class I ribozyme sequences for further analysis, we conducted a single additional round of selection (G7-ATP) that exploited the difference in catalytic activity between the dominant hammerhead sequences and the less common class I variants when incubated in the presence of ATP. G6 RNA was incubated for 10 min in the presence of 1 mM ATP under otherwise identical selection conditions. Class I ribozymes are expected to dominate under these new selection conditions because they show no catalytic inhibition in the presence of ATP. As before, the ribozyme's 5′-cleavage product was isolated by PAGE and used to produce the 'G7-ATP' RNA pool. Cloning and sequencing of this pool revealed that nearly 70% of the RNA pool consists of class I ribozymes, while the representation of hammerhead ribozymes dropped to ~25%. The additional class I sequences obtained from G7-ATP are new variants, except for a single repetition of the class I ribozyme v6 (Figure 5A).

The class I ribozyme v2 carries all the most frequently occurring nucleotides in both the N_9 and N_5 domains (Figure 6). Applicant further examined the class I v2 ribozyme by creating the bimolecular ribozyme arrangements termed 'v2 trans' (Figure 7). The v2 trans ribozyme displays catalytic activity. In addition, the new ribozyme cleaves the corresponding RNA substrate at the internucleotide linkage that immediately precedes the

site cleaved by the hammerhead ribozyme. The v2 trans ribozyme cleaves this new site with a k_{obs} of 0.02 min^{-1} using 1 μM enzyme and trace amounts of substrate in buffer B.

Ribozyme Engineering

Sequence, chemical and structural variants of Class I and Class II ribozymes can be engineered using the techniques shown above and known in the art to cleave a separate target RNA or DNA in *trans*. For example, the size of class I and class II ribozymes can, be reduced or increased using the techniques known in the art (Zaug *et al.*, 1986, *Nature*, 324, 429; Ruffner *et al.*, 1990, *Biochem.*, 29, 10695; Beaudry *et al.*, 1990, *Biochem.*, 29, 6534; McCall *et al.*, 1992, *Proc. Natl. Acad. Sci., USA.*, 89, 5710; Long *et al.*, 1994, Supra; Hendry *et al.*, 1994, *BBA* 1219, 405; Benseler *et al.*, 1993, *JACS*, 115, 8483; Thompson *et al.*, 1996, *Nucl. Acids Res.*, 24, 4401;Michels *et al.*, 1995, *Biochem.*, 34, 2965; Been *et al.*, 1992, *Biochem.*, 31, 11843; Guo *et al.*, 1995, *EMBO. J.*, 14, 368; Pan *et al.*, 1994, *Biochem.*, 33, 9561; Cech, 1992, *Curr. Op. Struc. Bio.*, 2, 605; Sugiyama *et al.*, 1996, *FEBS Lett.*, 392, 215; Beigelman *et al.*, 1994, *Bioorg. Med. Chem.*, 4, 1715; all are incorporated in its totality by reference herein) For example the aptamer domain (*e.g.*, ATP aptamer) of the ribozymes of either Class I or Class II may not be essential for catalytic activity and hence could be systematically reduced in size using a variety of methods known in the art, to the extent that the overall catalytic activity of the ribozyme is not significantly decreased.

Further rounds of *in vitro* selection strategies described herein and variations thereof can be readily used by a person skilled in the art to evolve additional

nucleic acid catalysts and such new catalysts are within the scope of the instant invention.

New self-cleaving ribozymes

The rAD-H2 construct was designed to allow the comprehensive screening of all possible sequence variants of the hammerhead catalytic core. Therefore, the allosteric delay strategy should give a distinct selective advantage to those ribozyme variants that remain active, yet benefit from allosteric inhibition during transcription. Interestingly, two new classes of self-cleaving ribozymes have emerged from the selection that are as active as AD-H2, but that do not undergo ATP-specific allosteric delay. This difference in allosteric inhibition was subsequently used to selectively enrich the RNA pool for class I ribozymes. Specifically, class I ribozymes represent less than 20% of the individual ribozymes that were recovered from the G6 RNA pool. Allosteric inhibition of the dominant hammerhead ribozymes by ATP during the preparation of the G7-ATP RNA pool created a drastic change in the composition of the ribozyme population. The G7-ATP pool consists of ~75% class I variants, thereby making further sequence analysis of this class proceed more efficiently.

Both class I and class II ribozymes have acquired additional mutations outside the N_9 and N_5 randomized domains. These position may be mutation 'hot spots', or may be infrequent but essential mutations that offer a significant selective advantage for those RNAs that have acquired them. Applicant has isolated 18 class I ribozyme variants that have considerable sequence variability, but that carry only a single mutation outside of the randomized N_9 and N_5 domains (Figure 5A). It is likely that most of these class I variants were individually repre-

sented in the original RNA pool and that each variant independently acquired the G to C mutation at position 28 of the aptamer during the *in vitro* selection process. None of the hammerhead ribozymes isolated from G6 carry aptamer mutations, suggesting that the mutations observed in the new ribozyme classes might be necessary for efficient catalytic function, and that these mutations may occur infrequently.

The class I ribozymes can be divided into separate ribozyme and substrate domains to create a functional bimolecular complex. This ribozyme presumably interacts with the substrate domain by forming base-paired regions that are analogous to helices I and II of the hammerhead ribozyme (Figure 1). Likewise, the substrate specificity of class I ribozymes can presumably be altered by changing the sequences of the substrate-binding arms to complement the sequence of the desired substrate molecule, as was achieved with the ribozyme v2 trans (Figure 7). Although the hammerhead and class I ribozymes cleave at different internucleotide linkages (Figure 1), both ribozymes appear to proceed by a similar chemical mechanism. The hammerhead ribozyme is known to produce a 2′,3′-cyclic phosphate at the terminus of the 5′-cleavage product, thereby leaving a 5′-hydroxyl terminus on the 3′-cleavage fragment. The 5′-cleavage products from both the hammerhead- and v2 trans-cleaved substrate RNAs migrate with the major products of alkaline-promoted substrate degradation that are terminated with 2′,3′-cyclic phosphates as opposed to the minor products that carry a 2′(3′)-phosphate. This is consistent with the possibility that, like the hammerhead, class I ribozymes utilize a transesterification mechanism to cleave the target RNA phosphodiester linkage. The similarity between the class I ribozymes and the hammerhead appear to end here, since removal of the

aptamer domain results in inactivation of the v2 ribozyme. Therefore, at least a portion of the mutant aptamer domain appears to be required for class I catalytic function.

The natural consensus sequence for the hammerhead catalytic core also was not improved upon by *in vitro* selection using a low Mg^{2+} concentration (buffer C). Here, the class I variant ribozymes come to dominate the G9-low RNA pool, however, the catalytic advantage of class I ribozymes compared to the hammerhead ribozymes that were isolated at G6 is subtle (Table 1). Although the class II ribozyme v1 displays catalytic rates that are similar to both the hammerhead and class I ribozymes in buffer B, and to class I ribozymes in buffer C (Table 1). However, this ribozyme or related variants do not comprise a major portion of either the G6 or the G9-low RNA pools. If the mutations acquired by the two representatives of class II are necessary for efficient catalytic function, then these ribozymes would have been present at a much lower frequency than either the hammerhead or class I ribozymes. As a result, this class of catalyst would not be expected to dominate the selected RNA pools due to their infrequent occurrence in the G0 and subsequent RNA pools.

Applicant has used ATP-controlled allosteric ribozymes to create self-cleaving hammerhead ribozymes that can be isolated as intact precursors from *in vitro* transcription reactions. Hammerhead ribozyme, as well as other ribozymes, could be designed that can be controlled by different allosteric effector molecules by judicious coupling of distinct aptamer and ribozyme domains. In the instant study, the ATP-dependent allosteric ribozyme design has been particularly beneficial for preparing unimolecular hammerhead ribozymes for use in kinetic analyses. Applicant has employed this concept of

allosteric delay in the design of a randomized RNA construct that was used to probe the catalytic fitness of the hammerhead ribozyme.

Using the allosteric delay strategy, Applicant found that the dominant class of ribozymes to emerge after six rounds of *in vitro* selection included sequences that matched the natural consensus of the catalytic core, including the re-identification of the variable-nucleotide site at position 7. This comprehensive screening of all possible sequence variations within the 14-nucleotide core region is expected to have simultaneously tested the catalytic fitness of nearly 270 million RNA molecules.

Finally, although the allosteric selection construct was heavily biased in favor of the hammerhead ribozyme, the selection produced two new ribozymes that cleave RNA with rates that are comparable to the allosteric hammerhead ribozymes. This finding, taken together with other reports of the *in vitro* selection of unique self-cleaving ribozymes, suggests that many novel ribozymes with similar catalytic activities have yet to be found. The application of *in vitro* selection using an unbiased starting pool of random-sequence RNA is likely to produce novel self-cleaving ribozymes with catalytic rates that meet or perhaps even exceed those of natural ribozymes.

Materials and Methods

Synthetic oligonucleotides and transcription-template constructs

Synthetic DNA and RNA oligonucleotides were prepared (Keck Biotechnology Resource Laboratory, Yale University) by standard solid-phase methods, purified by denaturing PAGE (8 M urea, 89 mM Tris-borate, 2 mM EDTA), and isolated by crush-soaking in 10 mM Tris-HCl (pH 7.5 at 23°C), 200 mM NaCl and 1 mM EDTA. The 2′-TBDMS group of the

synthetic RNA substrate (5′-GCCGUAGGUUGCCC) was removed by 24-hr treatment with triethylamine trihydrofluoride (15 µl per AU_{260} crude RNA). Purified RNA substrate was [5′-^{32}P]-labeled with T4 polynucleotide kinase and [γ-^{32}P]-ATP, then repurified by PAGE.

In vitro transcription

For each RNA sequence or RNA pool, 10 to 100 pmoles of DNA template was transcribed in buffer A (50 mM Tris-HCl (pH 7.5 at 23°C), 15 mM $MgCl_2$, 5 mM dithiothreitol and 2 mM spermidine) containing 2 mM each of the four ribonucleoside triphosphates. RNA synthesis was initiated by the addition of T7 RNA polymerase to a final concentration of 12 U μL^{-1} and incubated for 1 to 3 hours at 37°C. Internally-labeled transcripts were prepared by the inclusion of 20 µCi of [α-^{32}P]-UTP in the transcription reactions. Transcription products were separated by denaturing 10% PAGE, visualized by autoradiography or by electronic imaging (PhosphorImager, Molecular Dynamics) and the ribozymes were recovered from excised gel particles by crush-soaking. Concentrations of purified RNAs were established by liquid scintillation counting.

In vitro selection

G0 pool RNA was prepared by transcribing 100 pmoles of double-stranded DNA in a 100-µL reaction volume, corresponding to a population of rAD-H2 templates that is expected to include all possible sequence combinations within the catalytic core of the hammerhead ribozyme. This template DNA was prepared by extending 200 pmoles primer 2 (5′-GAATTCTAATAC-GACTCACTATAGGAAGAGATGGCGAC) in the presence of 200 pmoles of the oligonucleotide 5′-TTTGAGGCGACCTACCACTCTCGTGG$(N)_5$TTGCTGCGACCGAAGTCGCACAGTTTC-TTCCCAA$(N)_9$GTCGCCATCTCTTCC (where N indicates an equal mix

of the four nucleotides) with *Taq* polymerase under polymerase chain reaction (PCR) conditions. The PCR extension reaction was conducted in a total of 100 µL containing 0.05 U μL^{-1} *Taq* polymerase, 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3 at 23°C), 0.01 % gelatin, and 0.2 mM each dNTP for 1 cycle of 20 sec at 92°C, 20 sec at 50° C and 30 sec at 72°C.

For the initial round of selection, a total of 50 pmoles rAD-H2 RNA was incubated in a 50 µL reaction mixture containing 50 mM Tris-HCl (pH 7.5 at 23°C) and 20 mM $MgCl_2$ (buffer B) for 1 hr at room temperature. The reaction was terminated by the addition of an equal volume of PAGE loading buffer (8 M urea, 5 mM Tris-borate (pH 8.3 at 23°C), 0.3 M sucrose, 50 mM Na_2EDTA, 0.02% w/v xylene cyanol, and 0.02% w/v bromophenyl blue) and the products were separated by denaturing 10% PAGE. Reaction products were visualized by autoradiography, phosphorimage, or by UV shadowing. Great care was taken to optimize the separation between uncleaved precursor RNAs and the 5′ cleavage fragments. The cleaved ribozymes were isolated by crush/soaking the corresponding gel fragments and were concentrated by precipitation with ethanol. Recovered RNAs were reverse transcribed using 30 pmoles of primer 1 (5′-TTTGATGGCGACCTACCACTCTC-GTGG) in a 25 µL reaction containing 10 U μL^{-1} SuperScript reverse transcriptase (BRL) and incubated at 37°C for 30 min in the buffer supplied by the manufacturer. The resulting DNA was amplified by PCR as described above for 25 cycles. This entire process was repeated for addition selection rounds with some alterations. Specifically, transcription of subsequent generations was conducted using ~12 pmoles of PCR DNA from the preceding generation in a total of 50 µL as described above. RNA populations G3-G6 were twice

purified by PAGE to eliminate deletion mutants. After G3, only 50% of the cDNA from the selected RNA molecules was used for amplification by PCR. Finally, the reaction time for the sixth round of selection was reduced to 3 min.

The 'G7-ATP' pool was generated by selecting RNAs from the G6 pool that are active in the presence of ATP. This selection proceeded like that of the previous rounds, but the ribozyme selection reaction was conducted for 10 min in buffer B containing 1 mM ATP. The 'G9-low' pool was derived from G5 RNA, which was subjected to four additional rounds of selection using a ribozyme reaction buffer containing 50 mM Tris-HCl (pH 7.5 at 23°C), 250 mM KCl, and 2 mM $MgCl_2$ (buffer C). The low magnesium selection reactions were carried out at 23°C for 1 hr, or 10 min for the final round. Individuals from all populations of interest were analyzed by cloning (TA cloning kit, Invitrogen) and sequencing (ThermalSequenase Kit, Amersham).

Ribozyme assays

Self-cleaving ribozyme assays were conducted with internally-labeled precursor RNAs in buffers A, B or C as indicated for each experiment. Bimolecular ribozyme assays were conducted with trace amounts of [5′-^{32}P]-labeled substrate RNA and 1 μM of internally-labeled RNA enzyme. The results were analyzed by denaturing 10% PAGE and were visualized and analyzed by autoradiography or by PhosphorImager (Molecular Dynamics). For kinetic assays, a series of time points were made that best represented the initial rate of ribozyme cleavage. Catalytic rates (k_{obs}) were obtained by plotting the fraction of substrate cleaved versus time and establishing the slope of the line that represents the initial velocity of the reaction. For assays conducted in 1 mM ATP, the precursor RNA was

preincubated with ATP for 10 min in the absence of Mg^{2+}. This eliminated the burst kinetics that are observed with reactions that are initiated by the simultaneous addition of ATP and Mg^{2+}. Replicate experiments gave k_{obs} values that differed by less that 20% and the values reported are averages of two or more repetitions.

Cleavage sites for H1 and v2 trans ribozymes were determined by PAGE separation of ribozyme reactions using a partial alkaline digest of the synthetic substrate as a marker. The partial alkaline digest was made by incubation of a trace amount of [5'-^{32}P]-labeled substrate RNA in 100 mM NaOH for 5 min.

Diagnostic uses

Enzymatic nucleic acids of this invention may be used as diagnostic tools to examine genetic drift and mutations within diseased cells or to detect the presence of target RNA in a cell. The close relationship between ribozyme activity and the structure of the target RNA allows the detection of mutations in any region of the molecule which alters the base-pairing and three-dimensional structure of the target RNA. By using multiple ribozymes described in this invention, one may map nucleotide changes which are important to RNA structure and function *in vitro*, as well as in cells and tissues. Cleavage of target RNAs with ribozymes may be used to inhibit gene expression and define the role (essentially) of specified gene products in the progression of disease. In this manner, other genetic targets may be defined as important mediators of the disease. These experiments will lead to better treatment of the disease progression by affording the possibility of combinational therapies (e.g., multiple ribozymes targeted to different genes, ribozymes coupled with known small molecule inhibitors, or intermittent

treatment with combinations of ribozymes and/or other chemical or biological molecules). Other *in vitro* uses of ribozymes of this invention are well known in the art, and include detection of the presence of mRNAs associated with disease condition. Such RNA is detected by determining the presence of a cleavage product after treatment with a ribozyme using standard methodology.

In a specific example, ribozymes which can cleave only wild-type or mutant forms of the target RNA are used for the assay. The first ribozyme is used to identify wild-type RNA present in the sample and the second ribozyme will be used to identify mutant RNA in the sample. As reaction controls, synthetic substrates of both wild-type and mutant RNA will be cleaved by both ribozymes to demonstrate the relative ribozyme efficiencies in the reactions and the absence of cleavage of the "non-targeted" RNA species. The cleavage products from the synthetic substrates will also serve to generate size markers for the analysis of wild-type and mutant RNAs in the sample population. Thus each analysis will require two ribozymes, two substrates and one unknown sample which will be combined into six reactions. The presence of cleavage products will be determined using an RNAse protection assay so that full-length and cleavage fragments of each RNA can be analyzed in one lane of a polyacrylamide gel. It is not absolutely required to quantify the results to gain insight into the expression of mutant RNAs and putative risk of the desired phenotypic changes in target cells. The expression of mRNA whose protein product is implicated in the development of the phenotype is adequate to establish risk. If probes of comparable specific activity are used for both transcripts, then a qualitative comparison of RNA levels will be adequate and will decrease the cost of the initial

diagnosis. Higher mutant form to wild-type ratios will be correlated with higher risk whether RNA levels are compared qualitatively or quantitatively.

Additional Uses

Potential usefulness of sequence-specific enzymatic nucleic acid molecules of the instant invention might have many of the same applications for the study of RNA that DNA restriction endonucleases have for the study of DNA (Nathans *et al.*, 1975 *Ann. Rev. Biochem.* 44:273). For example, the pattern of restriction fragments could be used to establish sequence relationships between two related RNAs, and large RNAs could be specifically cleaved to fragments of a size more useful for study. The ability to engineer sequence specificity of the ribozyme is ideal for cleavage of RNAs of unknown sequence.

Other embodiments are within the following claims.

Table I: Cleavage Kinetics of Allosteric Ribozymes

	k_{obs} (min^{-1})			
	–	+	–	ATP
Construct	20	20	2	Mg^{2+} (mM)
AD-H1	0.18	–	0.011	
G6 pool	0.047	–	–	
AD-H2 (U)	0.068	0.00051	0.0051	
AD-H2 (C)	0.041	0.00050	0.0021	
class I (v1)	0.055	0.043	0.0032	
class I (v2)	0.070	0.053	0.011	
class II (v1)	0.054	0.016	0.012	

Table II

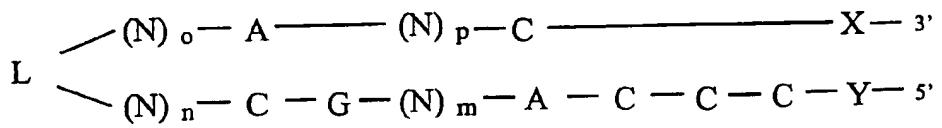
Table II: 2.5 μmol RNA Synthesis Cycle

Reagent	Equivalents	Amount	Wait Time*
Phosphoramidites	6.5	163 μL	2.5
S-Ethyl Tetrazole	23.8	238 μL	2.5
Acetic Anhydride	100	233 μL	5 sec
N-Methyl Imidazole	186	233 μL	5 sec
TCA	83.2	1.73 mL	21 sec
Iodine	8.0	1.18 mL	45 sec
Acetonitrile	NA	6.67 mL	NA

* Wait time does not include contact time during delivery.

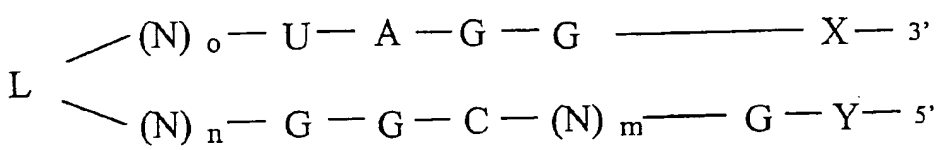
Claims

1. A nucleic acid molecule with an endonuclease activity having the formula I:



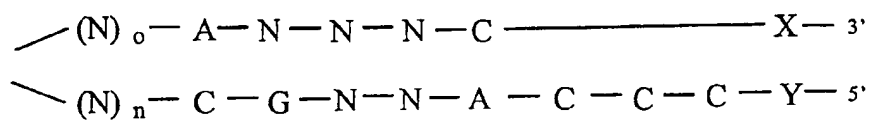
wherein, each N represents independently a nucleotide or a non-nucleotide linker, which may be same or different; X and Y are independent oligonucleotides of length sufficient to stably interact with a target nucleic acid molecule; o and n are integers greater than or equal to 1, wherein if (N)o and (N)n are nucleotides, (N)o and (N)n may interact by hydrogen bond interaction; p and m are selected independently from the integers 0, 1, 2, 3, 4 or 5; L is a linker which may be present or absent, wherein said linker, when present, is a nucleotide and/or a non-nucleotide linker, wherein said nucleotide linker comprises a single-stranded and/or double-stranded region; _____ represents a chemical linkage; and A, C and G represent adenosine, cytidine and guanosine nucleotides, respectively.

2. A nucleic acid molecule with catalytic activity having the formula II:



wherein, each N represents independently a nucleotide or a non-nucleotide linker, which may be same or different; X and Y are independent oligonucleotides of length sufficient to stably interact with a target nucleic acid molecule; o and n are integers greater than or equal to 1, wherein if $(N)_o$ and $(N)_n$ are nucleotides, (N)o and (N)n may interact by hydrogen bond interaction; m is selected independently from the integers 0, 1, 2, 3, 4 or 5; L is a linker which may be present or absent, wherein said linker, when present, is a nucleotide and/or a non-nucleotide linker, wherein said nucleotide linker comprises a single-stranded and/or double-stranded region; _____ represents a chemical linkage; and A, C, U and G represent adenosine, cytidine, uridine and guanosine nucleotides, respectively.

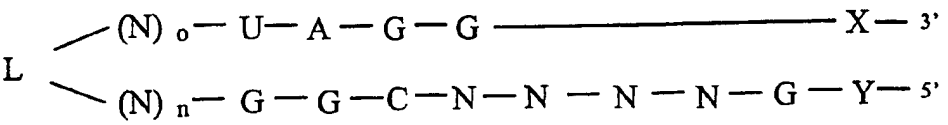
3. A nucleic acid molecule with catalytic activity having the formula **III**:



wherein, each N represents independently a nucleotide or a non-nucleotide linker, which may be same or different; X and Y are independent oligonucleotides of length sufficient to stably interact with a target nucleic acid molecule; o and n are integers greater than or equal to 1, wherein if $(N)_o$ and $(N)_n$ are nucleotides, (N)o and (N)n may interact by hydrogen bond interaction; L is a linker which may be present or absent, wherein said linker, when present, is a nucleotide and/or a non-nucleotide linker, wherein said nucleotide linker comprises a single-stranded and/or double-stranded region; _____ represents a

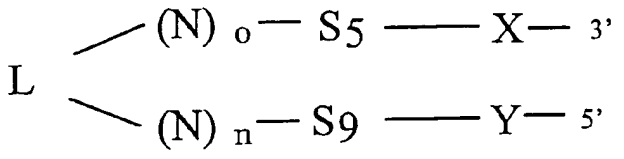
chemical linkage; and A, C and G represent adenosine, cytidine and guanosine nucleotides, respectively.

4. A nucleic acid molecule with catalytic activity having the formula **IV**:



wherein, each N represents independently a nucleotide or a non-nucleotide linker, which may be same or different; X and Y are independent oligonucleotides of length sufficient to stably interact with a target nucleic acid molecule; o and n are integers greater than or equal to 1, wherein if $(N)_o$ and $(N)_n$ are nucleotides, (N)o and (N)n may interact by hydrogen bond interaction; L is a linker which may be present or absent, wherein said linker, when present, is a nucleotide and/or a non-nucleotide linker, wherein said nucleotide linker comprises a single-stranded and/or double-stranded region; _____ represents a chemical linkage; and A, C, U and G represent adenosine, cytidine, uridine and guanosine nucleotides, respectively.

5. A nucleic acid molecule with catalytic activity having the formula V:



wherein, each N represents independently a nucleotide or a non-nucleotide linker, which may be same or different; X and Y are independent oligonucleotides of length sufficient to stably interact with a target nucleic acid molecule; o and n are integers greater than or equal to 1, wherein if $(N)_o$ and $(N)_n$ are nucleotides, $(N)_o$ and $(N)_n$ may interact by hydrogen bond interaction; L is a linker which may be present or absent, wherein said linker, when present, is a nucleotide and/or a non-nucleotide linker, wherein said nucleotide linker comprises a single-stranded and/or double-stranded region; _____ represents a chemical linkage; S_5 is an oligonucleotide containing a sequence selected from the group consisting of 5'-AUGUC-3', 5'-ACGUC-3', 5'-ACGGC-3', 5'-ACCUC-3', 5'-AAGGC-3', 5'-AUGGC-3', 5'-AUGCC-3', 5'-ACUCC-3', 5'-AUGAC-3', 5'-ACGAC-3', 5'-UUAGG-3', and 5'-CUAGG-3'; and S_9 is an oligonucleotide containing a sequence selected from the group consisting of 5'-CCCAGUGCC-3', 5'-CCCAGUGCA-3', 5'-CCCAAUGCA-3', 5'-CCCAAUGCC-3', 5'-CCCAAUGCU-3', 5'-CCCAUAGCA-3', 5'-CCCAACGCA-3', 5'-CCCACCGCA-3', 5'-CCCAGAGCA-3', 5'-CCCAACGCU-3', 5'-CCCAUUGCA-3', 5'-GUAGACGGA-3', and 5'-GGUUUCGGG-3'.

6 The nucleic acid molecules of any of claims 1-5 further comprises a cytidine residue immediately 3' of said $(N)_n$.

7. The nucleic acid molecules of any of claims 1-5, wherein said L is nucleotide linker.

8. The nucleic acid molecule of claim 7, wherein said nucleotide linker is a nucleic acid aptamer.

9. The nucleic acid molecule of claim 8, wherein said aptamer is an ATP aptamer.

10. The nucleic acid molecule of claim 6, wherein said L is nucleotide linker.

11. The nucleic acid molecule of claim 10, wherein said nucleotide linker is a nucleic acid aptamer.

12. The nucleic acid molecule of claim 11, wherein said aptamer is a ATP aptamer.

13. The nucleic acid molecule of any of claims 1-5, wherein said nucleic acid cleaves a separate nucleic acid molecule.

14. The nucleic acid molecule of claim 13, wherein said separate nucleic acid molecule is RNA.

15. The nucleic acid molecule of claim 13, wherein said nucleic acid comprises between 12 and 100 bases complementary to said separate nucleic acid molecule.

16. The nucleic acid molecule of claim 13, wherein said nucleic acid comprises between 14 and 24 bases complementary to said separate nucleic acid molecule.

17. A cell including the nucleic acid molecule of any of claims 1-5.

18. The cell of claim 17, wherein said cell is a mammalian cell.

19. The cell of claim 18, wherein said cell is a human cell.

20. An expression vector comprising nucleic acid sequence encoding at least one of the nucleic acid molecule of any of claims 1-5, in a manner which allows expression of that nucleic acid molecule.

21. A cell including the expression vector of claim 20.

22. The cell of claim 21, wherein said cell is a mammalian cell.

23. The cell of claim 21, wherein said cell is a human cell.

24. A pharmaceutical composition comprising the nucleic acid molecule of any of claims 1-5.

25. A method for modulating expression of a gene in a plant cell by administering to said cell the nucleic acid molecule of any of claims 1-5.

26. A method for modulating expression of gene in a mammalian cell by administering to said cell the nucleic acid molecule of any of claims 1-5.

27. A method of cleaving a separate nucleic acid comprising, contacting the nucleic acid molecule of any of claims 1-5 with said separate nucleic acid molecule under conditions suitable for the cleavage of said separate nucleic acid molecule.

28. The method of claim 27, wherein said cleavage is carried out in the presence of a divalent cation.

29. The method of claim 28, wherein said divalent cation is Mg^{2+}.

30. The nucleic acid molecule of claims 1-5, wherein said nucleic acid is chemically synthesized.

31. The expression vector of claim 19, wherein said vector comprises:

a) a transcription initiation region;

b) a transcription termination region;

c) a gene encoding at least one said nucleic acid molecule; and

wherein said gene is operably linked to said initiation region and said termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule.

32. The expression vector of claim 20, wherein said vector comprises:

a) a transcription initiation region;

b) a transcription termination region;

c) an open reading frame;

d) a gene encoding at least one said nucleic acid molecule, wherein said gene is operably linked to the 3'-end of said open reading frame; and

wherein said gene is operably linked to said initiation region, said open reading frame and said termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule.

33. The expression vector of claim 20, wherein said vector comprises:

a) a transcription initiation region;

b) a transcription termination region;

c) an intron;

d) a gene encoding at least one said nucleic acid molecule; and

wherein said gene is operably linked to said initiation region, said intron and said termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule.

34. The expression vector of claim 20, wherein said vector comprises:

a) a transcription initiation region;

b) a transcription termination region;

c) an intron;

d) an open reading frame;

e) a gene encoding at least one said nucleic acid molecule, wherein said gene is operably linked to the 3'-end of said open reading frame; and

wherein said gene is operably linked to said initiation region, said intron, said open reading frame and said termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule.

35. A method of selecting a nucleic acid with catalytic activity comprising the steps of:

a) providing a ligand binding nucleic acid region and a randomized nucleic acid region attached to said ligand binding nucleic acid region; and

b) selecting for nucleic acid molecules with said catalytic activity in the presence of said ligand.

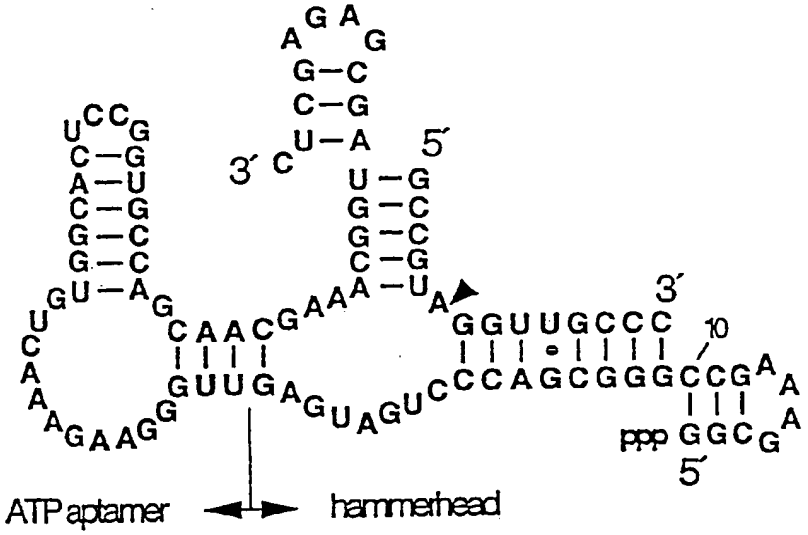
36. The method of claim 35, wherein said ligand binding region is an ATP aptamer.

37. The method of claim 36, wherein said ligand is ATP.

38. A nucleic acid molecule with catalytic activity selected using the method of claim 35.


Cleavage Site
Stem I
Stem III
Stem II
Loop II
Target
Ribozyme
5' - ..... N N N N U H N N N N N ..... - 3'
3' - ..... N' N' N' N' A15.1 A14 A13 G12 C3 U4 G5 A6 N7 G8 A9 N' N' N' N' N' ..... - 5'

Fig. 1A



H3

Fig. 1B

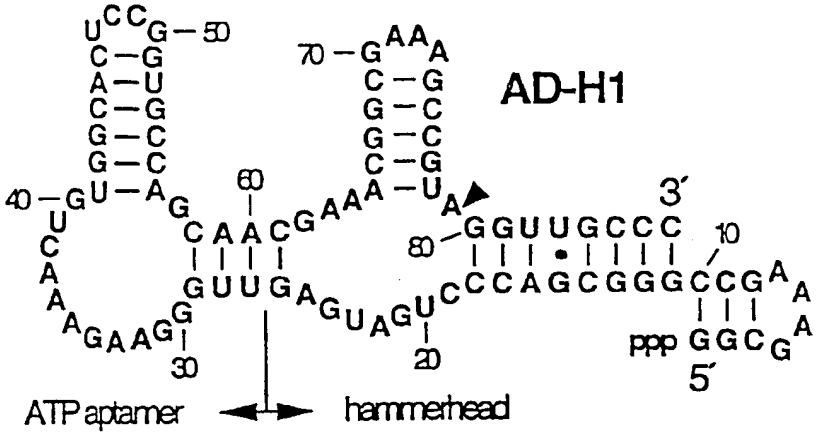


Fig. 2A

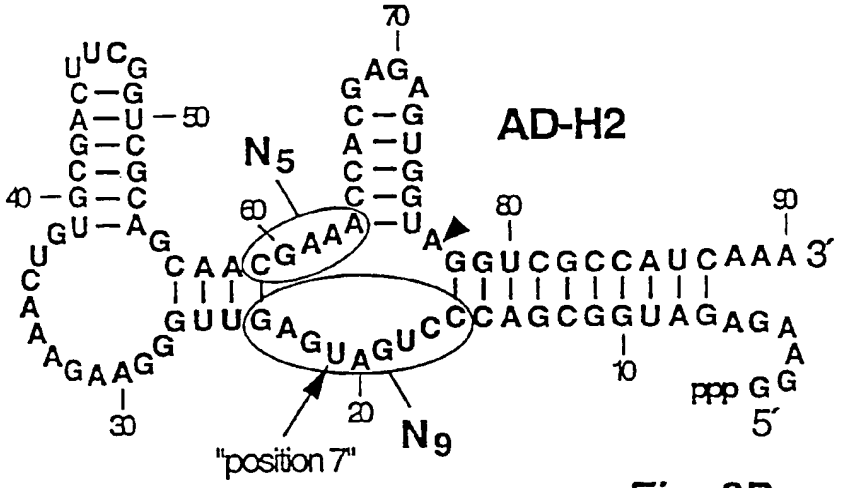


Fig. 2B

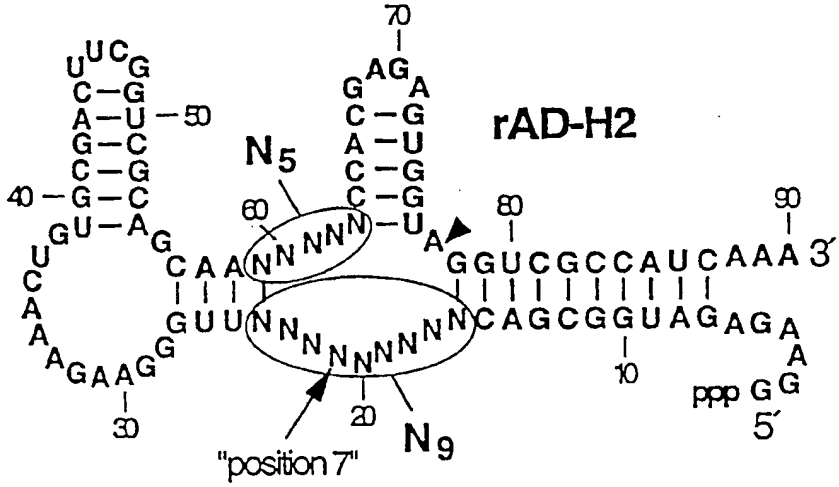


Fig. 2C

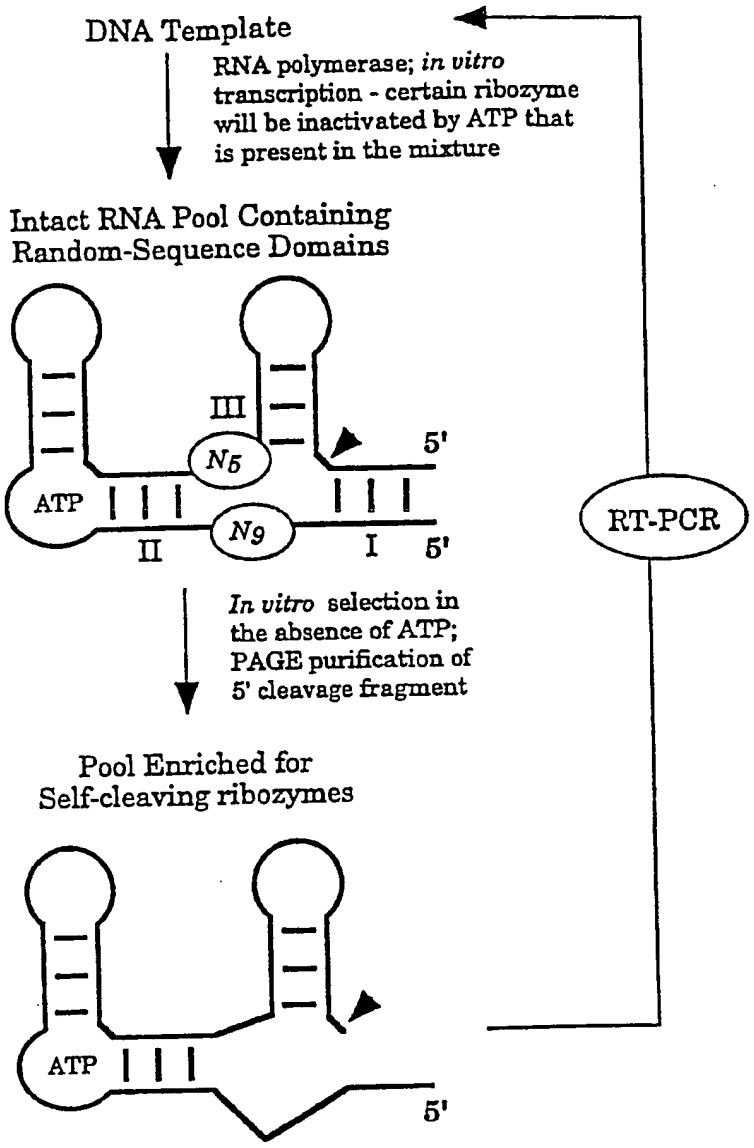

DNA Template
RNA polymerase; in vitro transcription - certain ribozyme will be inactivated by ATP that is present in the mixture
Intact RNA Pool Containing Random-Sequence Domains
III
N5
5'
ATP
N9
II
I
5'
RT-PCR
In vitro selection in the absence of ATP; PAGE purification of 5' cleavage fragment
Pool Enriched for Self-cleaving ribozymes
ATP
5'

Fig. 3

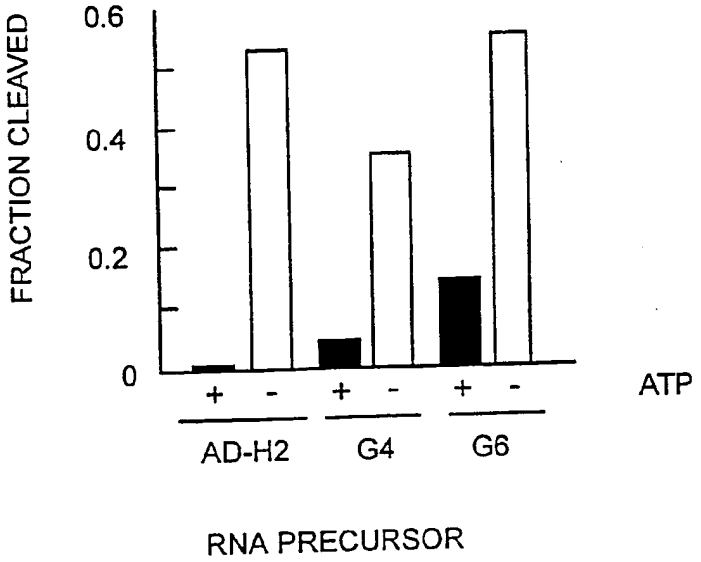


Fig. 4

Fig. 5A

Class I

		5′	N_9	N_5	3′
Generation 6	v1		CCCAGUGCC	AUGUC	
	v2		CCCAGUGCA	ACGUC	
	v3		CCCAGUGCA	ACGGC	
	v4		CCCAAUGCA	ACGUC	
	v5		CCCAAUGCC	ACCUC	
	v6		CCCAGUGCA	AAGGC	
	v7		CCCAAUGCU	AUGGC	
Generation 7-ATP	v6	(1)			
	v8		CCCAGUGCA	AUGCC	
	v9		CCCAUAGCA	AUGGC	
	v10		CCCAAUGCA	ACUCC	
	v11		CCCAACGCA	ACGGC	
	v12		CCCACCGCA	AUGAC	
	v13		CCCACCGCA	AUGGC	
	v14		CCCAAUGCC	AUGGC	
	v15		CCCAGAGCA	ACGAC	
	v16		CCCAACGCU	AUGGC	
	v17		CCCAUUGCA	ACGGC	
Generation 9 Low	v2	(3)			
	v4	(1)			
	v18		CCCAUAGCA	ACGAC	
	v19		CCCAGUGCA	ACGAC	

Fig. 5B

Class II

	5′	N_9	N_5	3′
v1		GUAGACGGA	UUAGG	
v2		GGUUUCGGG	CUAGG	

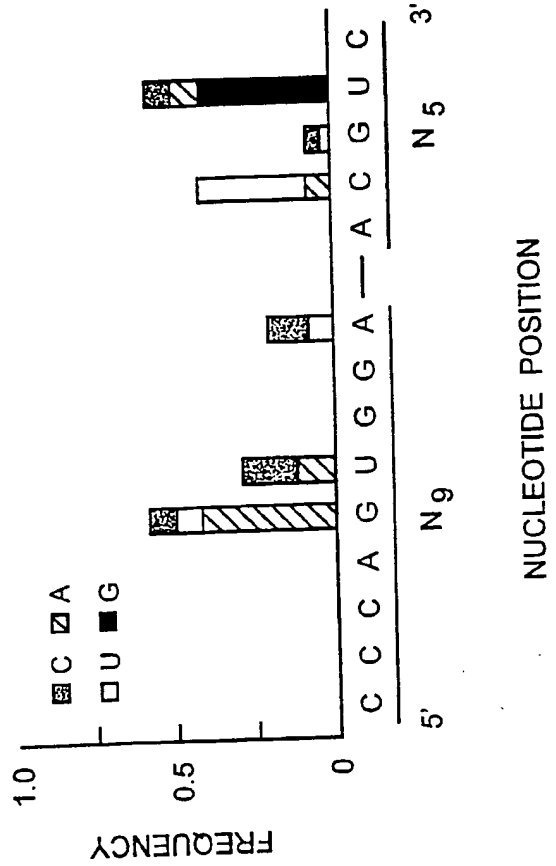

FREQUENCY
1.0
0.5
0
C A
U G
C C C A G U G G G A — A C G U C
5'
3'
N9
N5
NUCLEOTIDE POSITION

Fig. 6

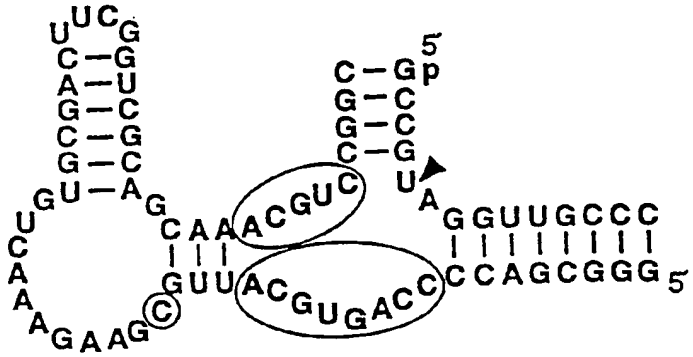


Fig. 7